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Glomerulonephritis induced in sheep by immunization with human glomerular basement membrane

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Glomerulonephritis induced in sheep by immunization with human glomerular basement membrane. The specificity of the anti-glomerular basement membrane (GBM) antibodies in experimental nephritis in sheep (Steblay's nephritis) was studied and compared with the specifity of antibodies in human anti-GBM nephritis (Goodpasture's syndrome). Sheep were injected monthly with isolated human GBM and antibody reactivities with isolated human and sheep GBM proteins were quantified with ELISA. Expectedly, the sheep had high titers of antibodies against several human GBM antigens. These antibodies remained for the most part in the circulation. In contrast, circulating antibody levels against sheep GBM antigens remained low for a long period of time, but a significant and progressive increase coincided with the development of acute nephritis. These antibodies accumulated in the kidneys of the nephritic sheep and could be eluted from diseased kidneys. They represent auto-antibodies immunologically cross–reacting with antigens of both sheep and human GBM. The specificity of auto-antibodies eluted from the kidneys was analyzed by immunoblotting and ELISA. The major populations reacted with one subunit, termed M2, of the globular domain of collagen IV. The same subunit contains the major antigen in Goodpasture's syndrome. It is concluded that the M2 subunit of the globular domain of collagen IV is recognized by IgG antibodies that primarily bind to the glomerular basement membrane in both Steblay's nephritis and Goodpasture's syndrome, indicating that it is a main nephritogen in both diseases.

Autoimmune glomerulonephritis due to production of antibody against glomerular basement membrane antigens was discovered experimentally by Steblay, who induced fatal glomerulonephritis in sheep by repeated injections of isolated heterologous glomerular basement membrane (GBM) suspended in adjuvant [1, 2]. Characteristic linear binding of sheep IgG along the GBM could be shown by immunofluorescence staining. Glomerulonephritis could be induced in healthy sheep both by passive transfer of serum antibodies and antibodies eluted from the kidneys of nephritic sheep [1–3] and by cross–circulation [4]. The pathogenetic role of the antibodies was thus established. Similarly it was shortly thereafter discovered that Goodpasture's syndrome in humans (glomerulonephritis and lung hemorrhage) is induced by anti-GBM antibody with the characteristic linear pattern of immunoglobulin deposition along the glomerular basement membrane, demonstrable in kidney biopsies [5, 6].

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Basement membranes are complex structures containing many potential immunogens that can induce an autoimmune response [7]. The major element is collagen IV which is composed of three chains (alpha 1 and alpha 2, type IV) with N-terminal, partly collagenous regions (7S domains), C-terminal noncollagenous globular domains (NC1 domains), and with an intervening major collagenous portion having a triple helical structure with interruptions, which are pepsin–sensitive [8]. In addition basement membranes contain the cell attachment protein laminin [9], entactin [10], and small amounts of proteoglycans [11]. GBM preparations will also contain small amounts of filtered plasma proteins and cell–derived constituents [7].

Recent research has shown that the antibodies in patients with Goodpasture's syndrome are directed against epitopes present in the collagenase–resistant globular domains of collagen IV [12–14] or more precisely, in a monomeric subunit, termed M2, of the globular domain [15]. Earlier studies in our laboratory indicated that the circulating antibody response in the artificially–induced sheep disease might be broader than in spontaneous human disease, but the nephritogens were not characterized at that time and eluted antibodies were not studied [16]. The present study, that was initiated to characterize these antigens and the tissue bound antibodies, indicates that one major probable nephritogen in the sheep model for antibody mediated glomerulonephritis is indeed very similar to that in Goodpasture's syndrome.

Methods

Induction of glomerulonephritis

Three one–and–a–half–year–old female sheep were immunized with 5 mg of lyophilized human glomerular basement membrane (H-GBM) suspended in saline and homogenized with an equal volume of Freund's Complete Adjuvant. Injections were done at several locations subcutaneously in the neck. Booster doses with 5 mg of H-GBM in Freund's Incomplete Adjuvant were given once a month over periods of five to thirteen months. Blood samples were drawn at intervals, at least monthly, and sera were analyzed for creatinine concentrations and used in immunological studies. Renal biopsy specimens were prepared and analyzed histopathologically and by immunofluorescence microscopy. In one sheep extensive histopathological studies of most organ systems were performed.

Laboratory methods

Serum creatinine was analyzed according to Heinegård and Tiderström [17]. Sheep IgG concentrations in serum and eluates were assayed by single radial immunodiffusion according to Mancini, Carbonara and Heremans [18]. Protein concentrations were determined according to Lowry et al [19].

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done as described by Laemmli [20], using 6 to 22% gradient gels.

Immunoblotting was performed as described by Burnette [21]. Proteins separated on SDS-PAGE were transferred by electrophoresis to nitrocellulose papers (Bio Rad Laboratories, Richmond, Virginia, USA). Bound proteins were immunostained by incubation of the papers overnight with serum or eluates from sheep followed by a one hour incubation with swine anti-sheep IgG, labelled with horseradish peroxidase (Dako, Copenhagen, Denmark). The peroxidase activity was visualized using the histochemical substrate 3,3′-diaminobenzidine–tetrahydrochloride (Fluka AG, Switzerland) [22].

Enzyme linked immunosorbent assay (ELISA)

ELISA was performed essentially as described before [23, 24], but the conditions used for coating were varied with the antigen. Antigens solubilized from GBM with bacterial collagenase as well as purified globular domains of collagen IV, and its isolated subunits M1, M2, and M3 were coated, 0.1 μg/ml, in 6 M guanidine-HCl, 0.05 M Tris-HCl, pH 7.5. Antigens solubilized with pepsin were coated, 0.5 μg/ml, in 0.1 M acetic acid. Pre-immune sera or eluates from normal sheep kidneys were used as negative controls. Longitudinal serum samples were analyzed on one immunoplate for each animal and antigen preparation, to avoid day to day variability with ELISA.

Inhibition experiments were performed by pre-incubating the diluted sera or eluates overnight at 4°C with the antigens (0 to 100 μg protein/ml) to be tested for their capacity to inhibit in the assay. Pre-incubated samples were then transferred to microtiter plates coated with the different antigens and analyzed as described elsewhere [23, 24].

Preparation of GBM antigens

Human and sheep GBM used for antigen preparations were isolated by sieving of kidney cortex and sonication of glomeruli as described before [24]. Proteinase inhibitors were included in all preparative steps, that is 5 mM N-ethylmaleimide, 5 mM benzamidine-HCl, 25 mM 6-amino–hexanoic acid, 10 mM EDTA, 1 mM phenylmethansulfonylfluoride. Human GBM used for immunization was, however, prepared without use of proteinase inhibitors.

Crude human and sheep GBM antigens were solubilized using bacterial collagenase (CLS, Worthington, Freehold, New Jersey, USA) which had been purified by the procedure described by Lee–Own and Anderson [25]. Digestion was carried out at 37°C, 16 hours with human or sheep GBM suspended in 0.05 M Hepes buffer, pH 7.45 with 0.01 mM calcium chloride and protease inhibitors, omitting EDTA, as described for the preparation of particulate GBM.

Pepsin–resistant human and sheep GBM collagen fragments were prepared as described before [16, 23]. In short, human and sheep GBM, respectively, were solubilized with pepsin. Type IV collagen triple helical fragments were precipitated with sodium chloride at acid pH and at neutral pH [16], essentially as described by Kresina and Miller [26] and Sage, Woodbury and Bornstein [27].

The globular domain (NC 1) of collagen IV was isolated from crude collagenase digests by a procedure involving ion exchange chromatography on DEAE cellulose followed by molecular sieving on Sephacryl S-200 [12, 28].

Monomeric subunits, M1, M2 and M3 of the globular domain were isolated from bovine GBM by reversed phase HPLC [15].

The 7S domain of collagen IV was isolated from human placenta according to Risteli et al [29].

Purified laminin was obtained from a basement membrane producing tumor of mice, that is, the Engelbreth–Holm–Swarm (EHS) sarcoma.

Elution of immunoglobulins from the kidneys

Kidney cortex (5 g of wet weight) from a normal sheep and from each of the nephritic sheep was homogenized and washed 20 times each with 50 ml of buffer, 0.05 M Tris-HCl, 0.15 M sodium chloride, pH 7.5 including proteinase inhibitors as described for the preparation of GBM. Insoluble material (1.27 to 1.79 g wet weight) was eluted with 20 ml of ice cold buffer, 0.1 M glycine, 0.5 M sodium chloride, pH 2.8 for 10 minutes and then with fresh buffer, pH 2.2, for 10 minutes. Supernatants were immediately adjusted to pH 7.4 to 7.8 with 3 M Tris-HCl. The pH 2.8 and pH 2.2 eluates from each kidney were pooled and proteins were precipitated with 50% (saturated) ammonium sulphate. The precipitate was dissolved in 1.5 ml, 0.05 M Tris-HCl, 0.15 M sodium chloride, 0.05% sodium azide, pH 7.5 and then dialysed against the same buffer.

Results

Case histories

Two of the three sheep (I and II, Table 1) developed rapidly progressive renal failure, documented by a progressive increase of serum creatinine levels, after five and 11 monthly injections of human particulate GBM (sheep I shown in Fig. 1). The third sheep (III, Table 1) developed proteinuria but serum creatinine levels remained constant. This sheep was sacrificed after 14 monthly injections of GBM. At autopsy the kidneys of all sheep were enlarged and showed peteccie. Other organs, including the lungs, appeared normal. Histopathological studies of the renal lesions revealed extracapillary proliferative glomerulonephritis in all glomerulae of sheep I and II, but focal segmental mesangial glomerulonephritis accompanied by focal sclerosis in sheep III. Immunofluorescence staining demonstrated a linear fixation of IgG and C3 along the glomerular basement membranes in all three sheep. According to these criteria, then, the three animals had developed an anti-GBM antibody glomerulonephritis. Most organs of sheep II were taken to histopathological studies, which disclosed moderate inflammatory cell infiltrates in the spleen and lymph nodes. The cervical lymph nodes, draining the sites of injections of GBM, had hyperplastic lymph follicles. The lungs showed occasional foci of granulomatous inflammatory changes. All other organs, except the kidneys, were normal. Proteins in urine were measured monthly in sheep II and III. Significant proteinuria was found to develop concurrent

Table 1. Induction and course of anti-GBM nephritis in sheep immunized with lyophilized particulate human GBM

	Immunization schedule		Development of uremia (serum creatinine >300 μmoliter/l	Renal morphology Light microscopy	Deposition of sheep IgG
	Initial dose Freunds complete adjuvant	Booster dose Freunds incomplete adjuvant			
Sheep I	5 mg	5 mg × 5 months	6 months	Extracapillary GN (100% crescents)	Linear, GBM
Sheep II	5 mg	5 mg × 10 months	11 months	Extracapillary GN (100% crescents)	Linear, GBM
Sheep III	5 mg	5 mg × 14 months	no uremia	Fokal GN, sclerosis (moderate)	Linear, GBM

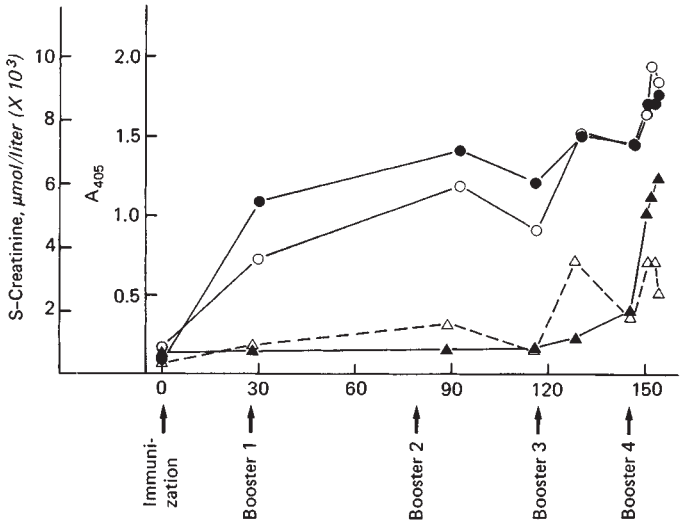

Fig. 1. *Relation between disease activity (serum creatinine) and anti-GBM antibody reactivities in sheep I, immunized with particulate human GBM.* Symbols are; (▲) serum creatinine μmol/liter; (●) antibodies against human GBM antigens solubilized with bacterial collagenase; (○) antibodies against globular domains of human collagen IV; (△) antibodies against globular domains of sheep collagen IV.

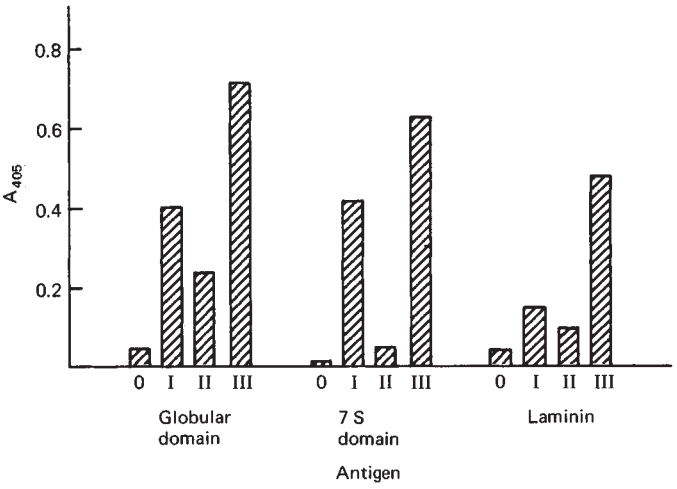

Fig. 2. *Enzyme-linked immunosorbent assay of serum antibodies at the time of sacrifice in three sheep immunized with particulate human GBM.* Serum dilutions of 1:20,000.

with the rapid increase of the serum creatinine concentration in sheep II and during the last month in sheep III.

Circulating serum antibodies

The three sheep had produced high titers of antibodies against several components of GBM. Collagenous components, liberated by pepsin digestion of human GBM as well as non-collagenous components, liberated by collagenase digestion of human GBM, reacted strongly in ELISA (data not shown). Better indications of the presence of circulating antibodies with particular specificity was obtained by strong reactions in ELISA against purified GBM constituents, that is the globular domain of human collagen IV from GBM, laminin from the mouse EHS sarcoma and 7S domain from human placenta (Fig. 2). Interestingly, titers against collagenous (pepsin resistant) and noncollagenous (collagenase resistant) sheep GBM proteins (not shown in the figure) and against isolated globular domains of sheep collagen IV (Fig. 1) were very low during the period of immunization. The titers, however, increased concurrently with serum creatinine in the two animals which developed rapidly progressive glomerulonephritis (Fig. 1).

Cross-reactivity of antibodies eluted from the kidneys

If auto-immunity has a role in heterologously induced sheep nephritis one would expect that autoantibodies, that is, antibody populations with specificity against sheep GBM epitopes would accumulate in the kidneys and be depleted in the circulation. Linear binding of sheep IgG along the GBM of the nephritic sheep and the low titers of anti-sheep GBM antibodies found in serum indicated that such an accumulation of specific auto-antibodies may occur. In support, antibodies could be recovered from the kidneys by elution at acid pH. The yields of eluted sheep IgG, quantified according to Mancini, Carbonara and Heremans [18] were 40 μg (sheep I), 250 μg (sheep II) and 100 μg (sheep III) per g wet weight kidney tissue.

Titration experiments of sera and eluates showed that antibodies with specificity for sheep GBM components were preferentially bound in the kidneys (Fig. 3). Based on IgG levels, reactivities against human GBM components were very similar for eluate and serum, but the eluate showed a 50 to 200 times stronger reaction with sheep GBM than did the serum.

Further experiments using competitive ELISA showed that antibody populations present in the eluates cross-reacted with collagenase-resistant and, partly, with pepsin-resistant antigens of both human and sheep GBM (results found in sheep I shown in Fig. 4). These results indicate that antibody popula-

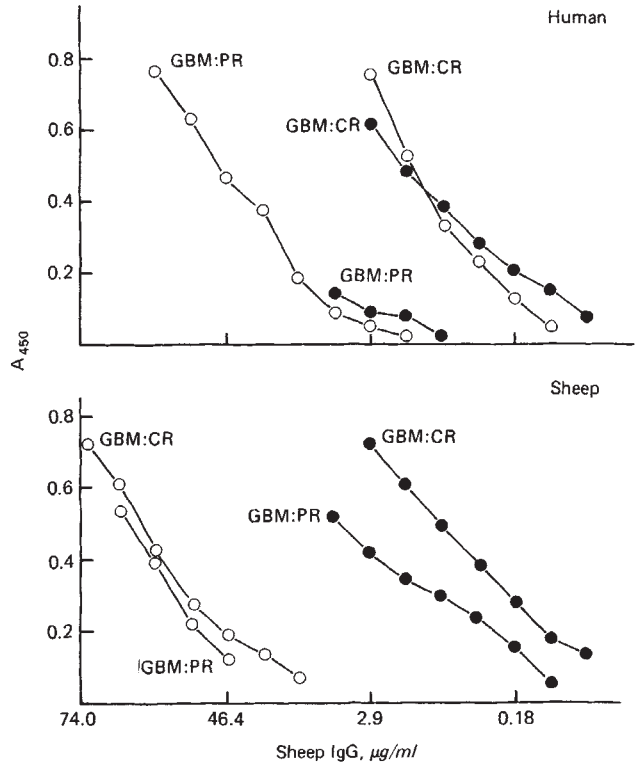

Fig. 3. *Titration of antibodies eluated from the kidney cortex of a sheep with anti-GBM nephritis, induced by repeated immunizations with particulate human GBM.* Antibody reactivity (A 450) in kidney eluates (●) and serum (○) expressed per weight IgG.

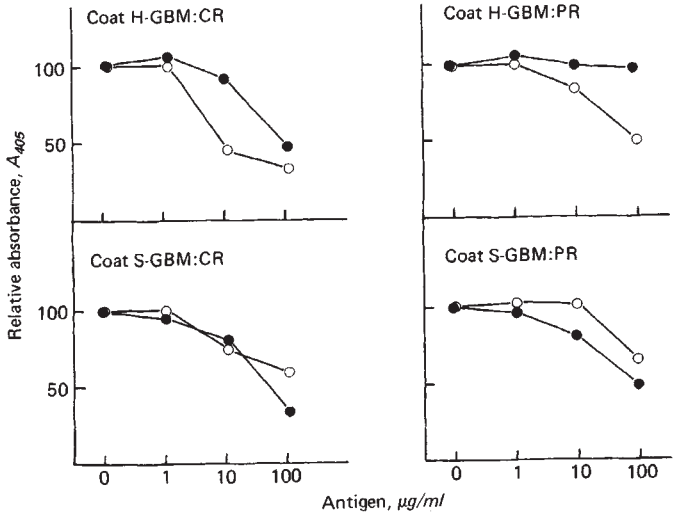

Fig. 4. *Competitive enzyme–linked immunosorbent assay: Antibodies eluted from kidney cortex of sheep 1, immunized with human GBM, were preincubated with solubilized GBM antigens and then tested for residual reactivity with different antigens by ELISA.* Coated antigens are: collagenase–resistant human (H-GBM:CR) and sheep (S-GBM:CR) antigens, and Pepsin–resistant human (H-GBM:PR) and sheep (S-GBM:PR) antigens. Inhibiting antigens are: (GBM:CR) sheep (●) and human (○) collagenase–resistant GBM antigens, and (GBM:PR) sheep (●) and human (○) pepsin–resistant GBM antigens.

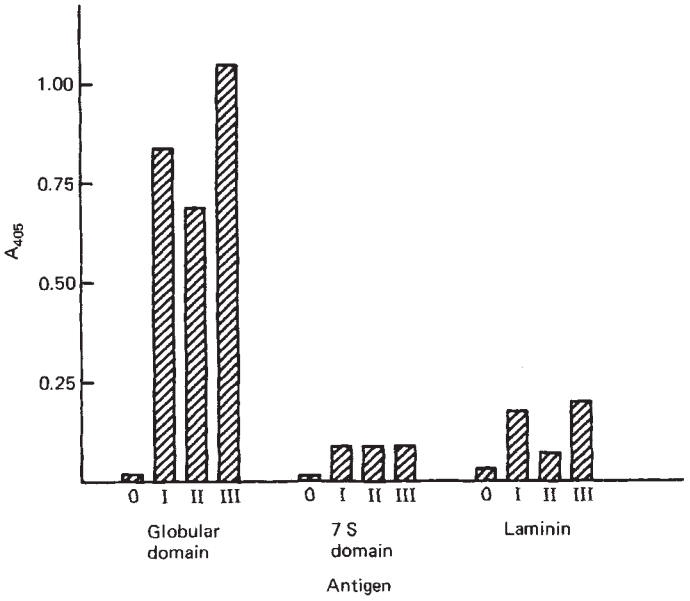

Fig. 5. *Enzyme–linked immunosorbent assay of antibodies eluted from kidney cortex of three sheep with anti-GBM nephritis, induced by immunization with human GBM.*

tions, cross–reacting with both human and sheep antigens, were the ones that had accumulated in the kidneys of the sheep.

Fine specificity of antibodies eluted from kidneys

Eluted immunoglobulins reacted with high titers against globular domains of human collagen IV and with considerably lower titers against human 7S domains and laminin (Fig. 5). The specificities of the eluted antibodies were further investigated by immunoblotting experiments. Sheep GBM components, solubilized by digestion with bacterial collagenase, were separated by electrophoresis on SDS-PAGE. The proteins were then transferred electrophoretically to nitrocellulose papers and immunostained with gammaglobulins eluted from the kidneys of the three nephritic sheep. In addition, for control, transblotted proteins were immunostained with antibodies from a patient with Goodpasture's syndrome. Eluates from nephritic sheep almost exclusively stained human and sheep GBM proteins identified as monomeric and dimeric peptides derived from the globular domain of collagen IV. The staining pattern using antibodies from the human patient was identical, whereas an eluate from a normal sheep showed no staining (Fig. 6).

Antibodies from Goodpasture patients react only with monomeric and dimeric subunits, termed M2 and D 2, of the globular domain [15]. Antibodies eluted from the kidneys of the three sheep were tested in ELISA for their reactivity with the different monomeric subunits (M1,M2,M3) isolated from bovine GBM [15]. Interestingly, the eluates from all three sheep reacted mainly with the M2 subunit of the globular domain (Fig. 7). Bovine M1, M2 and M3 were used because of ease of preparation and availability. Indirect evidence, that sheep globular domains also contain these M1, M2 and M3 subunits was obtained by inhibition and immunoblotting experiments. Thus the reactivity of specific rabbit antisera against bovine M1, M2 and M3 could each be completely inhibited using sheep globular

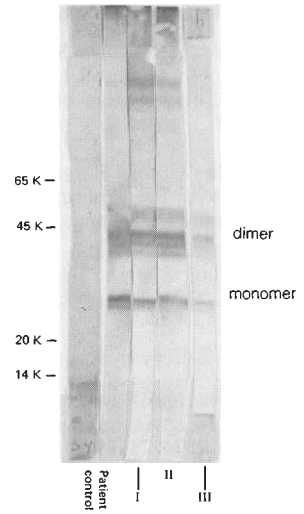

Fig. 6. *Immunoblotting: similar reactivity of antibodies from a patient with Goodpasture's syndrome (patient) and antibodies eluted from kidney cortex of three nephritic sheep (I,II and III) that were immunized with human GBM.* An eluate from a non-immunized sheep showed no reaction (control).

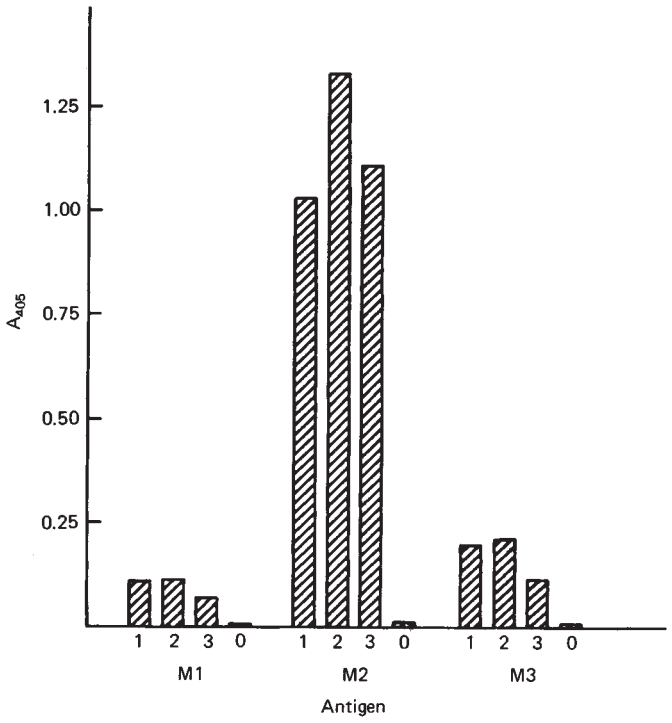

Fig. 7. *Enzyme–linked immunosorbent assay of antibodies eluted from kidney cortex of three sheep (1,2,3) with anti-GBM nephritis and a non-immunized control (○).* The antigens tested were different subunits (M1, M2 and M3) of the globular domain of bovine collagen IV.

domains as an antigen in competitive ELISA (not shown). Furthermore, these antibovine M1, M2 and M3 antisera stained collagenase–resistant sheep GBM products on immunoblotting having the mobility of globular domain monomers and dimers on SDS-PAGE (not shown). It was also shown, that the reactivity of a Goodpasture serum with M2 could be completely inhibited using purified sheep globular domain monomers as an antigen.

In conclusion, the immunostaining experiments, the solid phase binding assay (ELISA) and the inhibition experiments showed, that a major population of antibodies in eluates from kidneys of nephritic sheep recognized the same antigen, present in both human and sheep GBM, as did antibodies from patients with Goodpasture's syndrome.

Discussion

Although there is general agreement that antibody induces the disease in different forms of anti-GBM nephritis, there is controversy as to the fine specificity of these nephrotoxic antibodies [29]. We have recently shown that a major antibody population in patients with Goodpasture's syndrome recognizes a specific antigen, termed Goodpasture antigen [12]. The epitope(s) was (were) subsequently shown to be localized in one of the subunits (M2) of the globular domain of collagen IV [15]. In the present study, sheep that were immunized with heterologous (human) GBM produced an antibody population that recognized the same antigen (M2) of GBM. Several features indicated that these antibodies were autoreactive and the ones responsible for the development of nephritis.

The sheep responded to immunization with human GBM by developing a nephritis that appeared to run a two–phase course. No nephritis was evident for a long time although the serum had high titers of antibodies to heterologous (human) GBM antigens, shown by ELISA. In the second phase an augmented response to the challenge occurred, indicated by accelerating renal damage with rapidly increasing serum creatinine levels. The antibody titers against several human GBM antigens also increased progressively and hypergammaglobulinemia and proteinuria became prominent. The response to homologous (sheep) antigens was different. Only low levels of serum antibodies were found in the first phase, but in the last few days of culminating disease, an increase of the titers in serum was observed. A similar phenomenon has been described to occur after nephrectomy in both animals and man [30]. There are two possible explanations for the increase of circulating antibody in the final stage. First, with advancing damage renal plasma flow will decrease and glomerular capillary areas will successively be eliminated. Secondly, an augmented general antibody synthesis may include auto-antibodies. Available antigenic sites may then become saturated and antibodies will remain in circulation. In support large amounts of anti-sheep GBM antibodies could be eluted from the kidneys. Titration of serum and eluates by ELISA indicated that antibodies against homologous (sheep) antigens, presumably auto-antibody, had been more efficiently eliminated from the circulation and bound in the kidneys than had antibodies against heterologous (human) antigens (Fig. 3). Antibodies eluated from the kidney of the nephritic sheep reacted both with human and sheep GBM antigens by ELISA. Further studies using competitive ELISA showed that the reactivities with sheep antigens could be inhibited completely with either human or sheep GBM products. Eluates thus contained populations of antibodies, that cross–reacted with antigenic determinants found in both human and in sheep GBM. Eluted antibodies furthermore immuno-

stained digestion products of human and sheep GBM with the same mobility on SDS-PAGE, again confirming their cross–reactivity with heterologous and homologous GBM determinants. The fact that immuno-staining was observed with proteins derived from the globular domain of human, bovine and sheep collagen IV and that antibodies from a patient with Goodpasture's syndrome stained identical proteins provides strong indication, that the major nephritogen in Steblay nephritis and human Goodpasture's syndrome is the same. This hypothesis was further strengthened by results obtained by ELISA using isolated subunits of the globular domain of collagen IV as antigens. Both sheep antibody and Goodpasture antibody reacted best with the M2 subunit (Fig. 6).

Based on results with indirect immunofluorescence microscopy, it has previously been suggested that the antibodies in Steblay nephritis and Goodpasture's syndrome are similar [31], since antibodies eluted form the kidneys of nephritic sheep reacted in the same locus as did Goodpasture antibodies. Moreover, the sheep antibody inhibited the reaction of Goodpasture antibody with GBM [31]. The present study, in support, provides definite chemical evidence that Steblay and Goodpasture antibodies recognize the same GBM antigen.

Steblay observed, that the nephrotoxic antibodies in the sheep model were neither species nor organ specific. Indirect immunofluorescence microscopy showed that antibodies eluted from the kidneys of nephritic sheep reacted with basement membrane of all species and organs tested [32, 33]. Adsorption of eluates with sheep or human GBM or lung basement membrane preparations, removed the antibodies. Immunization of sheep with either human or sheep glomerular or human (but not sheep) lung basement membrane induced identical diseases [33]. The most likely explanation to these findings is that the antibodies react with the nephritogenic M2 subunit of collagen IV, which is a normal component of several basement membranes [7, 14].

It is not known why the GBM is a primary target or why the antibodies only cause nephritis in sheep while lung involvement is frequent in human disease. It has recently been demonstrated [28], that the epitope reacting with Goodpasture antibodies is sequestered in the hexameric globular domain of collagen IV. The epitope can be exposed by dissociation of the hexamers by treatment with guanidine-HCl of dilute acid in a reversible process [28]. It is conceivable, then, that some interference with the organisation of basement membrane components is a pre-requisite for antibody binding also in vivo. Furthermore, variable contents of globular domains and of unmasked epitopes in different basement membranes, may determine the in vivo localization of auto-antibody.

In the present study the sheep, in addition to the anti-M2 antibodies, produced small amounts of auto-antibody (that is, kidney–binding antibody) reactive with purified M1, M2 and laminin, which are normal components of basement membranes. Similarly, anti-laminin antibodies are occasionally found in patients with Goodpasture's syndrome [14, 34].

Antibodies against the 7S domain of collagen IV were present in serum but only in low amounts in eluates of the kidneys of the three sheep. These antibodies, therefore, probably had no role in the disease.

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